

*Preferential Binding of Solvent Components
to Protein In Mixed Water–Organic Solvent Systems†*

HIDEO INOUE‡ and SERGE N. TIMASHEFF

*Pioneering Research Laboratory, United States Department of Agriculture and
Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts
02154, U.S.A.*

Introduction

Recent studies, using the methods of partial specific volume,⁽¹⁾ isopiestic vapor pressure equilibrium⁽²⁾ and differential refractometry,⁽³⁾ have shown that many proteins bind preferentially the salt components in aqueous guanidine hydrochloride, if the reference state is chosen as that of equal molality of the salt on the two sides of the membrane. Light scattering and refractive index increment measurements^(4, 5) have shown that an organic solvent, 2-chloroethanol interacts with bovine serum albumin and β -lactoglobulin A in a more complicated manner. At low alcohol concentration, these proteins interact preferentially with 2-chloroethanol, and become preferentially hydrated at high concentrations of alcohol. While guanidine hydrochloride is a structure breaker, 2-chloroethanol is considered as a structure-forming agent.^(6, 7) The conformation of β -lactoglobulin A, for example, is changed from the native structure to one much richer in α -helix as the concentration of 2-chloroethanol increases from 10 to 20% by volume.⁽⁵⁾ It seemed of interest, therefore, to examine the relation between the helix-forming ability of some organic solvents and their preferential interaction with proteins in mixed water–organic solvent systems. For this purpose, the systems

† This work was supported in part by the National Institutes of Health, Grant No. GM14603 and the National Science Foundation, Grant No. GB 5186.

‡ Permanent Address: Shionogi Research Laboratory, Shionogi and Co., Ltd., Fukushima-ku, Osaka, Japan. This work was carried out during the tenure of a Resident Research Associateship within the Agricultural Research Service.

water-ethylene glycol and water-methoxyethanol were investigated, using β -lactoglobulin A as protein.

Theoretical

In any thermodynamic study of macromolecule-solvent interactions we determine the chemical potential of the macromolecular component in equilibrium with solvent components. Thus, in light scattering, for example, we measure the concentration fluctuations of the macromolecular component within a volume element of the solution. This is depicted schematically in Fig. 1, where the arrows indicate the fluctuations of particles within, into and out of a volume element. The kinetic units themselves must be defined now as macromolecules interacting with solvent components. Such a fluctuating, kinetic unit is depicted on the right-hand side of Fig. 1, where the numbers refer to the components. Here we use the

FLUCTUATIONS

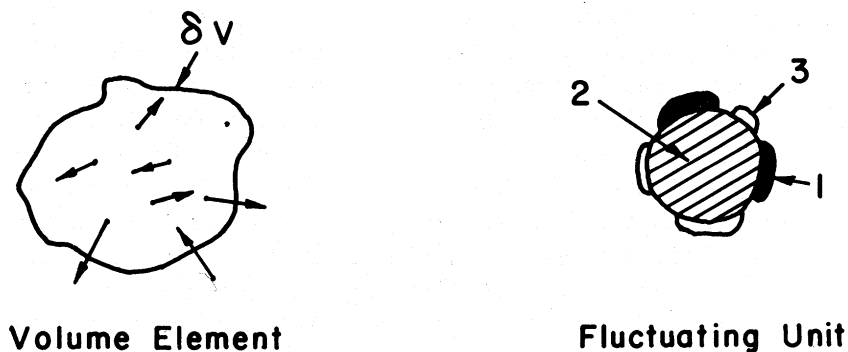


FIG. 1. Schematic representation of the phenomena measured in thermodynamic experiments on multicomponent interacting systems.

notation of Scatchard⁽⁸⁾ and Stockmayer,⁽⁹⁾ in which the macromolecular solute is component 2, water is component 1 and the organic solvent is component 3. We may write out the specific equation for differential refractometry. Expressing the concentrations on the molal scale, the preferential binding to a macromolecular component is related directly to the difference between the refractive index increments measured in turn on

systems in which the chemical potential and the molality of component 3 are kept identical in the protein solution and the reference solvent:

$$\begin{aligned} \left(\frac{\partial m_3}{\partial m_2}\right)_{T,p,\mu_3} &= \left\{ \left(\frac{\partial n}{\partial m_2}\right)_{T,p,\mu_3} - \left(\frac{\partial n}{\partial m_2}\right)_{T,p,m_3} \right\} / \left(\frac{\partial n}{\partial m_3}\right)_{T,p,m_2} \\ \left(\frac{\partial m_1}{\partial m_2}\right)_{T,p,\mu_1} &= -\frac{m_1}{m_3} \left(\frac{\partial m_3}{\partial m_2}\right)_{T,p,\mu_3} \end{aligned} \quad (1)$$

In this equation, n is the refractive index, m_i is the molal concentration of component i (moles per 1000 g of the principal solvent, or component 1). T is the thermodynamic temperature, p is the pressure, and μ_i is the chemical potential. In practice it is easier to measure the concentration in mass per volume units. Therefore, changing the concentration units from molality to grams per ml of solution, and extrapolating the concentration of component 2 to zero, we obtain

$$\begin{aligned} \frac{M_3}{M_2} \left(\frac{\partial m_3}{\partial m_2}\right)_{T,p,\mu_3}^0 &= \left(\frac{\partial g_3}{\partial g_2}\right)_{T,p,\mu_3}^0 \\ &= \frac{1}{(1 - \bar{V}_3 C_3)} \left\{ \left(\frac{\partial n}{\partial C_2}\right)_{T,p,\mu_3} - \left(\frac{\partial n}{\partial C_2}\right)_{T,p,m_3} \right\} / \left(\frac{\partial n}{\partial C_3}\right)_{T,p,m_2} \end{aligned} \quad (2)$$

where M is the molecular weight, g_i is the concentration of component i in grams per gram of component 1, \bar{V} is the partial specific volume measured in the usual way, and C_i is the concentration of component i in grams per ml of solution; the superscript 0 indicates extrapolation to zero of the protein concentration. While the definition of $(\partial n / \partial C_2)_{T,p,\mu_3}$ requires that all measurements be carried out under a hydrostatic head equal to the osmotic pressure of the solution, in practice, it is much simpler to do the measurements in a state of dialysis equilibrium, i.e. at constant T, μ_1, μ_3 . The error introduced by the practical approximation is quite minor.⁽¹⁰⁾ The amount of preferential binding, $(\partial m_3 / \partial m_2)_{T,\mu_1,\mu_3}$ or $(\partial g_3 / \partial g_2)_{T,\mu_1,\mu_3}$ is defined as zero when the amount of third component per 1000 g, or per gram, of water, respectively, is identical on both sides of the membrane at osmotic equilibrium. On the other hand, when we define as zero preferential binding the state in which the amount of third component per ml of solution is identical on both sides of the dialysis membrane, the preferential binding is expressed by $(\partial C_3 / \partial C_2)_{T,\mu_1,\mu_3}$ and is given by equation (3):^(3, 11)

$$\left(\frac{\partial C_3}{\partial C_2}\right)_{T,\mu_1,\mu_3} = \left\{ \left(\frac{\partial n}{\partial C_2}\right)_{T,\mu_1,\mu_3} - \left(\frac{\partial n}{\partial C_2}\right)_{T,p,C_3} \right\} / \left(\frac{\partial n}{\partial C_3}\right)_{T,p,C_2} \quad (3)$$

These two definitions of preferential binding are related by an expression^(3, 12) which reduces to equation (4) when C_2 is extrapolated to zero:

$$\left(\frac{\partial g_3}{\partial g_2}\right)_{T, \mu_1, \mu_3}^0 = \frac{g_3}{(\bar{V}_1)_{T, p, m_2} C_3} \left[\left(\frac{\partial C_3}{\partial C_2}\right)_{T, \mu_1, \mu_3}^0 + C_3(\bar{V}_2)_{T, p, m_3} \right] \quad (4)$$

Experimental

The proteins used were β -lactoglobulin A (β -Lg A), lysozyme, bovine serum albumin (BSA) and insulin. The concentrations of protein solutions were measured on a Zeiss PMQ II spectrophotometer. All protein solutions used included 0.02 M NaCl and 0.01 M HCl. The organic solvents, 2-chloroethanol, ethylene glycol and methoxyethanol were doubly distilled immediately before use.

The refractive index increments of the proteins were measured on a Brice differential refractometer⁽¹³⁾ at three different conditions, i.e. (1) keeping the chemical potential of the solvent components identical in solvent and solution, (2) keeping the molality of organic solvent identical, and (3) keeping the molarity of organic solvent identical. The details of these methods are described elsewhere.^(5, 14)

Light scattering measurements were carried out on the β -Lg A solutions in water-2-chloroethanol and water-methoxyethanol mixtures, using a Brice photometer.⁽¹⁵⁾ All measurements were done at constant molality of component 3, using the previously described technique.⁽⁵⁾

Results

The results of the preferential interaction of the organic solvent component to β -Lg A, measured on the molal, $(\partial g_3/\partial g_2)_{T, \mu_1, \mu_3}$, scale in mixtures of water with 2-chloroethanol, methoxyethanol and ethylene glycol are shown in Fig. 2.

At low concentrations of 2-chloroethanol, there is an evident preferential interaction of the organic component with the protein. This interaction increases with an increase in the alcohol concentration reaching a maximum at about 40 volume percent. Above this chloroethanol concentration, it decreases monotonely to negative apparent absorption of chloroethanol, i.e. to an apparent preferential interaction with water, after passing through zero at about 70 volume percent. Above this concentration, β -Lg A is

preferentially hydrated, so that at 80% chloroethanol, the observed preferential interaction is that of 0.2 g of water bound preferentially to 1 g of protein. The corresponding effect is quite different in the ethylene glycol and methoxyethanol systems. In the latter two solvent systems there is little, if any, preferential interaction below 30–40 volume percent; above this solvent composition, the organic solvent becomes progressively more bound to protein up to the highest composition studied. The results of refractometric measurements, carried out on a molal basis on lysozyme,

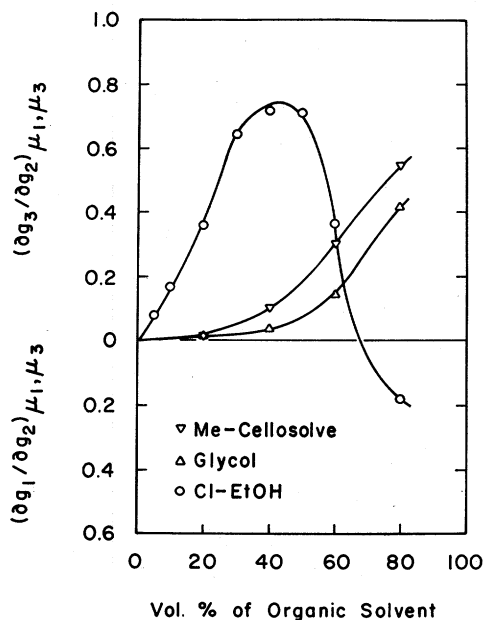


FIG. 2. Preferential interaction of β -lactoglobulin A with solvent components (expressed as grams solvent bound to 1 g of protein) in the systems water–2-chloroethanol (○), water–methoxyethanol (▽) and water–ethylene glycol (△).

BSA and insulin in the water–2-chloroethanol system indicates that an effect similar to that obtained with β -Lg A is found with these proteins; this suggests that the observed pattern is a property of the solvent and not the protein.⁽¹⁴⁾

All three organic solvents used are known to affect protein conformation. The helix inducing effect of the three solvents is shown for β -Lg A in

Fig. 3, where the change in the Moffitt-Yang b_0 parameter is presented as a function of increasing amounts of the organic component. Since b_0 is regarded to reflect principally the degree of α -helicity in a protein, the transitions shown on Fig. 3 represent conformational changes from the native globular structure to a denatured one, rich in α -helix. 2-Chloroethanol is a strong helix-inducing agent.^(6, 7, 16) When this solvent is present in amounts as small as 10% by volume, conformational changes are induced which progress with increasing contents of this material.^(5, 7) Both methoxyethanol and ethylene glycol are weaker helix-inducing agents.^(14, 17, 18) In the case of β -Lg A no structural changes occur at low

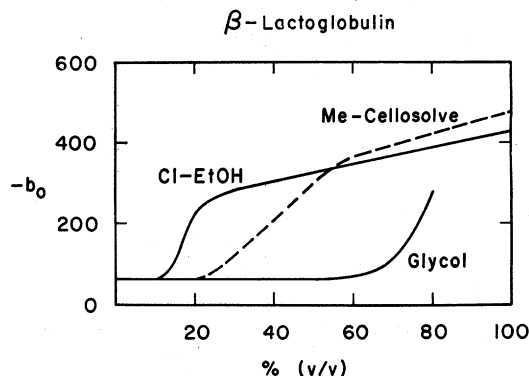


FIG. 3. Conformational changes induced in β -lactoglobulin A by increasing amounts of 2-chloroethanol, methoxyethanol and ethylene glycol.

organic solvent contents; above 30–60% organic component, the apparent helix content of the protein increases. The relative affinities of the protein for the three solvents, and the order in which they induce changes in the optical rotatory parameters of β -Lg A,^(5, 14, 16) suggest that preferential binding of the organic component and conformational changes may occur in parallel fashion.

In this respect the decrease in preferential binding of 2-chloroethanol above a 40% composition (Fig. 2) appears at first to be puzzling. It should be recalled, however, that the measured entity is not absolute binding but preferential interaction with solvent components relative to the bulk solvent composition. Thus, at 80% 2-chloroethanol, we are comparing the solvent composition in the immediate domain of the protein molecule to

a bulk solvent which has only 20% water. The structure of globular proteins in the native state is such that most polar residues are on the surface and probably hydrated, while the non-polar ones are in the hydrophobic interior of the molecule. When the protein unfolds as a result of the action of a denaturant such as 2-chloroethanol, few new polar regions become exposed. The originally hydrated regions, however, tend to remain so even at high organic solvent contents. Therefore, the total amount of water held by the protein will not change significantly with a change in solvent composition. This results in an increase in the relative amount of water in the immediate vicinity of the protein as the solvent composition changes to progressively smaller contents of water. These considerations permit us to separate the observed preferential binding of component 3 $(\partial m_3/\partial m_2)_{\text{app}}$ into two contributions—that due to the actual binding of component 3 as its concentration increases $(\partial m_3/\partial m_2)_{\text{true}}$ and that due to the tightly held water of hydration, $(\partial m_3/\partial m_2)_{\text{H}_2\text{O}}$:

$$\left(\frac{\partial m_3}{\partial m_2}\right)_{\text{H}_2\text{O}} = -\frac{m_3}{m_1} \left(\frac{\partial m_1}{\partial m_2}\right)_{T, p, \mu_1} \quad (5)$$

From equation (5) it is evident that this last effect will always make a negative contribution to the binding expressed in terms of interaction with the organic component. As (m_3/m_1) increases, this term becomes progressively larger even though $(\partial m_1/\partial m_2)_{T, p, \mu_1}$ remains constant. The magnitude of the tightly held water of hydration was calculated, assuming a constant value of 400 water molecules bound per β -Lg A molecule, i.e. 40% hydration which is consistent with small angle X-ray scattering results,⁽¹⁹⁾ and the results are shown on Fig. 4. It is evident that when the constant hydration of the protein is taken into account, the apparent negative binding of 2-chloroethanol at 80% composition turns out to be simply a reflection of the contribution of equation (5). When the observed binding is corrected for this contribution, we obtain the true interaction of the protein with the organic component, shown by the upper dashed line of Fig. 4. It is found, therefore, that as the chloroethanol concentration increases and the protein becomes progressively more helical, the organic solvent component is indeed progressively bound to the newly exposed nonpolar residues of the protein just as is the case of the weaker denaturants, ethylene glycol and methoxyethanol. The fact that the last two solvents exhibit no maximum in their interaction with the protein simply reflects their weaker action as structure-altering entities.

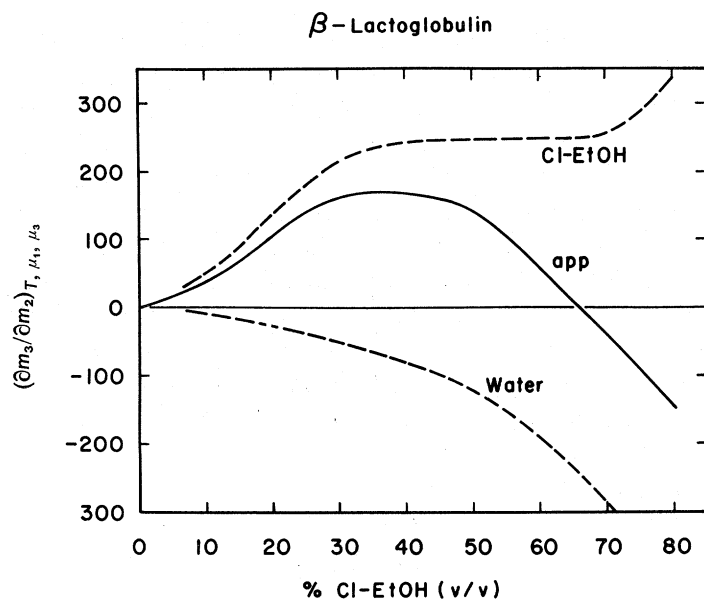


FIG. 4. Contributions of the binding of water and 2-chloroethanol to the net experimentally observed preferential interaction (relative to bulk solvent composition) of 2-chloroethanol with β -lactoglobulin A.

Summary

The preferential interaction of a protein with one of the solvent components in a mixed solvent can be detected by comparison of the refractive index increment before and after redistribution of solvent components across a membrane impermeable to the macromolecule, if the two components of the mixed solvent have non-identical refractive indices. Light scattering measurements and a proper application of multi-component theory also allow the determination of the preferential binding of solvent components to a protein.

We have investigated the preferential interaction of β -lactoglobulin A with solvent components in mixtures of water with 2-chloroethanol, ethylene glycol or methoxyethanol by the refractive index increment method and in part by the light scattering method. Results obtained from these methods show good agreement. The preferential binding of solvent components to various proteins, namely lysozyme, bovine serum albumin

and insulin in a mixed water-2-chloroethanol system, has also been investigated. These results are compared with those of conformational transition studies carried out in the same systems.

References

1. KIELLEY, W. W. and HARRINGTON, W. F., *Biochim. Biophys. Acta* **41**, 401 (1960).
2. HADE, E. P. K. and TANFORD, C., *J. Am. Chem. Soc.* **89**, 5034 (1967).
3. NOELKEN, M. E. and TIMASHEFF, S. N., *J. Biol. Chem.* **242**, 5080 (1967).
4. STAUFF, J. and MEHROTRA, K. N., *Kolloid-Z.* **176**, 1 (1961).
5. INOUE, H. and TIMASHEFF, S. N., *J. Am. Chem. Soc.* **90**, 1890 (1968).
6. DOTY, P., *Rev. Mod. Physics* **31**, 107 (1959).
7. CALLAGHAN, P. and MARTIN, N. H., *Biochem. J.*, **83**, 144 (1962).
8. SCATCHARD, G., *J. Am. Chem. Soc.* **68**, 2315 (1946).
9. STOCKMAYER, W. H., *J. Chem. Phys.* **18**, 58 (1950).
10. STIGTER, D., *J. Phys. Chem.* **64**, 842 (1960).
11. VRIJ, A. and OVERBEEK, J. TH. G., *J. Colloid Sci.* **17**, 570 (1962).
12. INOUE, H. and TIMASHEFF, S. N., *Adv. in Chem.*, **84**, 12 (1968).
13. BRICE, B. A. and HALWER, M., *J. Opt. Soc. Am.* **41**, 1033 (1951).
14. TIMASHEFF, S. N. and INOUE, H., *Biochemistry*, **7**, 2501 (1968).
15. BRICE, B. A., HALWER, M. and SPEISER, R., *J. Opt. Soc. Am.* **40**, 768 (1950).
16. TANFORD, C. and DE, P. K., *J. Biol. Chem.* **236**, 1711 (1961).
17. TANFORD, C., BUCKLEY, C. E., III, DE, P. K. and LIVELY, E. P., *J. Biol. Chem.* **237**, 1168 (1962).
18. KIENTZ, M. L. and BIGELOW, C. C., *Biochemistry* **5**, 3494 (1966).
19. WITZ, J., TIMASHEFF, S. N. and LUZZATI, V., *J. Am. Chem. Soc.* **86**, 168 (1964).